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DNA encoding ovine adenovirus (OAV 287) and its use as a viral vector

Technical Field

5 The present invention relates to a new full length genomic clone derived from a benign adenovirus (OAV287) isolated from sheep in Australia. The present invention also relates to new viral vectors derived from the benign ovine adenovirus and also relates to the use of these vectors for the delivery and expression of nucleic acid sequences encoding functional RNA molecules or  
10 polypeptides to animals.

Background of the Invention

Diseases caused by infectious agents and parasite infestations cause health problems and production losses in domestic animals but for many infectious agents no  
15 vaccine exists. Consequently, there are major research efforts worldwide to develop new vaccines which can protect against disease.

While some protective antigens from infectious agents and parasites have been identified, their  
20 successful use as vaccines requires the development of systems which can effectively deliver the antigen to the host. A variety of recombinant gene expression vectors derived principally from the pox virus family have been employed as these are generally of low pathogenicity.  
25 Expression of the foreign protein following infection by the recombinant viral vector may stimulate a protective immune response in the host.

However, no viral vector has all the attributes desirable for all situations. Some vectors are better  
30 suited to particular tasks than others because of their biological properties. For example, it has often proved difficult to stimulate an effective mucosal immune response which can protect against disease. In humans, adenoviruses have been given orally to vaccinate against  
35 respiratory disease (1). As this involves protection at mucosal surfaces adenoviruses clearly have potential in

this regard. Human adenovirus vectors have also been used to deliver genes to muscle (2) and other tissues. Although adenoviruses do not generally integrate their DNA into the cellular genome, nevertheless, the DNA persists and long term protein expression is observed. Expression of an appropriate antigen from such cells can generate a systemic immune response which may be protective against the homologous disease causing agent.

Known adenovirus genomes are linear double-stranded DNA molecules which have an inverted terminal repeat sequence (ITR) at each end and a protein covalently bound to the 5'-terminal C residue (3). The genome sequence and structure has now been completely determined for human adenoviruses types 2, 5, 12 and 40 and partially for numerous others, including some animal isolates (see Genebank and EMBL Nucleic Acid databases). Human adenovirus type 2 was the first genome to be sequenced but broadly speaking its genome arrangement is conserved among other characterized adenoviruses i.e. early regions E1-E4 and the structural protein homologues can be recognized in similar locations in the genome. In particular, the E1A/E1B region is located at the left hand end of the genome and region E4 is always located at the right hand end of the genome. Early region E3 is always located between the genes for structural proteins pVIII and fiber, although its size and complexity varies between species e.g. from 3kb with at least 10 open reading frames in human adenoviruses to approximately 0.7kb with only two significant open reading frames in murine adenovirus (4, 5). E3 is a key region for the construction of recombinant viruses as it is non-essential for replication in vitro (6). The late, L region is expressed from the major late promoter, MLP and complex splicing generates families of mRNAs which code for most of the structural viral proteins. Proteins IVa2 and IX appear to have their own promoters.

Although there are some human viral vectors available for medical use there are few animal viral vectors suitable for use in veterinary applications. In order to obtain a more suitable animal viral vector the present inventors have purified an ovine adenovirus (OAV287) isolated from sheep in Western Australia. This ovine adenovirus is serologically related to bovine adenovirus type 7 but is genetically distinct from the bovine adenoviruses and other Australian ovine isolates, as shown by comparisons between the ovine and bovine adenoviruses, based on restriction enzyme profiles (8). The genome arrangement of the virus according to the present invention varies significantly from all other known adenoviruses. The adenoviral DNA molecule of the present invention is suitable for use in viral vectors capable of expressing a variety of polypeptides when used for veterinary applications.

#### Summary of the Invention

546 <sup>53</sup> ~~34~~ According to a first aspect, the present invention consists in an isolated DNA molecule comprising a nucleic acid sequence encoding the genome of ovine adenovirus (OAV287) substantially as shown in Figure 1 or a functionally equivalent nucleic acid sequence. Preferably, the nucleic acid sequence encoding the genome of the adenovirus is substantially as shown in Figure 1.

In a further preferred embodiment of the first aspect of the present invention, the DNA molecule comprises a nucleic acid sequence encoding the genome of ovine adenovirus (OAV287) wherein a portion of the adenoviral genome not essential for the maintenance or viability of the native adenovirus deleted or altered.

In a second aspect, the present invention consists in a DNA molecule including at least a fifteen nucleic acid base sequence being substantially unique to the ovine adenovirus (OAV287) nucleic acid sequence shown in Figure 1. In a preferred embodiment of the second aspect of the

present invention, the at least fifteen nucleic acid base sequence encodes a functional element of ovine adenovirus (OAV287). Preferably, the functional element is selected from the group consisting of promoter, gene, inverted terminal repeat, viral packaging signal and RNA processing signal. The inverted terminal repeat of ovine adenovirus (OAV287) comprises the first 46 nucleic acid bases from the 5' end of each strand of the double stranded DNA genome of the virus.

In a third aspect, the present invention consists in a plasmid including the DNA molecule of the first or second aspects of the present invention. Preferably, the plasmid includes the DNA molecule of the first aspect of the present invention wherein the nucleic acid sequence encoding the adenoviral genome is linked to a nucleic acid sequence encoding an origin of replication and a further nucleic acid encoding a marker. Preferably, the nucleic acid sequence encoding the marker encodes for resistance to an antimicrobial agent. More preferably the antimicrobial agent is ampicillin.

In a further preferred embodiment of the third aspect of the present invention, sequences encoding inverted terminal repeats of the adenovirus are joined.

In a fourth aspect, the present invention consists in a viral vector comprising the DNA molecule of the first aspect of the present invention and at least one nucleic acid sequence encoding a non-adenoviral polypeptide or polypeptides.

Preferably, nucleic acid sequence encoding the non-adenoviral polypeptide or polypeptides is derived from bacteria, viruses, parasites or eukaryotes. More preferably, the non-adenoviral polypeptide is rotavirus VP7sc antigen, the parasite polypeptide is *Trichostrongylus colubriformis* 17kD antigen, the *Taenia ovis* 45W antigen or the PM95 antigen from *Lucilia cuprina*.

In another form, the present invention consists in a viral vector comprising the DNA molecule of the first aspect of the present invention and at least one nucleic acid sequence encoding a functional RNA molecule. It will be appreciated by one skilled in the art that a functional RNA molecule can include a messenger RNA molecule, an antisense RNA molecule or a ribozyme.

In a fifth aspect, the present invention consists in a method of delivering a DNA molecule having a nucleic acid sequence encoding a non-adenoviral polypeptide or polypeptides to a target cell comprising infecting the target cell with a viral vector according to the fourth aspect of the present invention such that the DNA molecule encoding the polypeptide or polypeptides is expressed and the polypeptide or polypeptides is produced by the target cell.

In a sixth aspect, the present invention consists in a method for delivering a DNA molecule having a nucleic acid sequence encoding a non-adenoviral polypeptide or polypeptides to an animal comprising administering to the animal a viral vector according to the fourth aspect of the present invention such that the viral vector infects at least one cell of the animal and the infected cell expresses the DNA molecule encoding the polypeptide or polypeptides and produces the polypeptide or polypeptides. Preferably the animal is a grazing animal and more preferably the grazing animal is a sheep.

In another form, the present invention consists in a method for delivering a DNA molecule having a nucleic acid sequence encoding a functional RNA molecule to an animal comprising administering to the animal a viral vector of the fourth aspect of the present invention having a nucleic acid sequence encoding a functional RNA molecule such that the viral vector infects at least one cell of the animal and the infected cell expresses the DNA

molecule encoding the functional RNA molecule and produces the functional RNA molecule.

As used herein the term "functionally equivalent nucleic acid sequence" is intended to cover minor variations in the ovine adenovirus (OAV287) DNA molecule which, due to degeneracy in the DNA code, does not result in the molecule encoding different viral polypeptides. Further, this term is intended to cover alterations in the DNA code which lead to changes in the encoded polypeptides, but in which such changes do not substantially affect the biological activities of these viral polypeptides.

As used herein the term "functional element" is intended to cover nucleic acid sequences that encode promoters, genes, inverted terminal repeats, viral packaging signals and RNA processing signals. It will be appreciated by one skilled in the art that unique sequences from ovine adenovirus (OAV287) that encode these functional elements may be useful in other systems including plasmids and non-ovine adenoviral vectors.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will be described with reference to the following examples and the accompanying drawings.

#### 25 4 Brief Description of the Drawings

Sub 1 Figure 1 is the nucleic acid sequence of the OAV287 genome beginning at base 1 of the left-hand ITR.

Figure 2 shows the arrangement of OAV287 genes based on homologies detected with Ad2. Regions with question marks are tentative identifications because of the lack of obvious homology.

Figure 3 indicates the major open reading frames in the proposed E1 region of OAV287. Asterisks show the location of possible initiation codons. A previously unidentified gene (p28kD) which codes for a processed structural protein is encoded on the complementary strand.

Figure 4 shows open reading frames in the region of the OAV287 expected to contain E3. However, E3 is missing as the gap between the pVIII and fiber genes is only 197 nucleotides. The site at which the ApaI/NotI polylinker was later inserted is indicated.

Figure 5 shows the major open reading frames in the probable E3 region of OAV287. Asterisks show the location of potential initiation codons. The SalI site which was modified by end-filling and re-ligation and the alternative site at which a polylinker sequence was later inserted into the genome without loss of infectivity is indicated.

Figure 6 is a scheme describing the construction of a plasmid (pOAV287Cm) containing a full-length clone of the OAV287 genome with pACYC184 sequences inserted in the SalI site. Filled in regions show OAV287 sequences. Cross-hatched sequences are derived from plasmids pUC13 or Bluescribe M13+ (Amp<sup>R</sup>), stippled regions from pSELECT (Tet<sup>R</sup>) and open regions from pACYC184(Cm<sup>R</sup>). Only the key restriction sites used for plasmid construction are indicated.

Figure 7 shows a map of the plasmids pOAV100, pOAV200, pOAV600 and pOAV600S. Arrowheads indicate the ITRs and the approximate location of the major late promoter (MLP). The mutated SalI site and sites at which the ApaI/NotI polylinker sequences were inserted are indicated. Light hatching signifies modified Bluescribe sequences inserted in the KpnI site. Linear, infectious genomes (dark hatching) are released by digestion with KpnI.

Figure 8 shows the results of screening ovine adenoviruses OAV100 and OAV200 rescued by transfection of recombinant plasmids pOAV100 and pOAV200 into CSL503 cells. Portions of the genome spanning (A) the mutated SphI site in OAV100 and (B) the ApaI/EcoRV/NotI polylinker insertion site in OAV200 were amplified by PCR together

with the corresponding regions from wild-type OAV287. The products were digested with SphI (A, lanes 3 & 5) and ApaI, EcoRV or NotI (B, lanes 3-5, and 8-10, respectively). (U) indicates undigested samples.

5 ~~Sub B-35~~ Figure 9 is a map of a plasmid pMT used for the assembly of gene expression cassettes. Fragments containing the OAV287 major late promoter and tripartite leader sequences are linked and precede a multiple cloning site for the insertion of genes of interest. A tandem polyadenylation signal (AATAAA) follows.

10 Figure 10 shows a summary of recombinant viruses which have been rescued from the corresponding infectious plasmids and the gene expression cassettes they carry. Cassettes were inserted into the OAV genome between the  
15 pVIII and fibre genes as indicated.

Figure 11 shows the expression of (A) the *T. ovis* 45W and *L. cuprina* PM95 antigens in CSL503 cells following infection of these cells with OAV205 and OAV210 viruses, respectively and (B) VP7sc expression in CSL503 and bovine  
20 nasal turbinate cells following infection with virus OAV204. (I) Infected cells (U) Uninfected cells. (M) indicates marker proteins of the sizes shown.

Figure 12 shows expression of VP7sc in (A) CSL503 cells and (B) rabbit kidney and bovine nasal turbinate  
25 cells following infection with OAV206 virus. (I) Infected cells. (U) uninfected cells. (M) indicates marker proteins of the sizes shown.

02-12-84 ~~Sub B-35~~  
Description of the Invention

METHODS

30 Growth and Purification of OAV287

The virus, isolated from sheep in 1985, was obtained from R.L. Peet, Animal Health Laboratory, Department of Agriculture, Western Australia. The virus isolate was grown in sheep foetal lung cells (line CSL503) and twice  
35 plaque-purified under solid overlay before stocks were prepared. Virus was purified from CSL503 cells as



described previously (18, 22). DNA was extracted from the virus by digestion with proteinase K (23).

#### Cloning of Genome Fragments

5 Molecular techniques for manipulation, modification and transformation of plasmid DNA which were used in the work described below are described in (9) and similar publications. OAV287 DNA was digested with various restriction endonucleases including BamHI, SphI, SmaI and SalI to deduce the location of these sites (18).

10 The adenovirus genome has a protein covalently linked to each end of the linear dsDNA (24). The BamHI A and D fragments of approximately 8kb and 4kb, respectively, were identified as the terminal genomic fragments because their migration into agarose gels was  
15 dependent on the pre-digestion of viral DNA with proteinase K. The internal BamHI fragments B, C, E and F<sub>2</sub> estimated at 6.2, 5.1, 3.4 and 1.1kb in size respectively, were separated on an agarose gel, recovered and cloned into BamHI-digested pUC13 using standard ligation and  
20 transformation procedures (9). To clone the terminal BamHI A and D fragments, viral DNA (10µg) was digested with proteinase K (50µg/ml in 10mM Tris/HCL, pH8.0, containing 1mM EDTA and 0.5% SDS) at 65°C for 60min to remove the terminal protein. The DNA was extracted twice  
25 with phenol/chloroform, once with ether and recovered by ethanol precipitation. The 3'ends (of unknown sequence) were then digested exo-nucleolytically with T<sub>4</sub> DNA polymerase (5 units, Toyobo, Tokyo, Japan) in the presence of dATP (100µM) in buffer containing Tris HCL (50mM),  
30 pH8.0, MgCl<sub>2</sub> (7mM), 2-mercaptoethanol (7mM) and BSA (10µg/ml) for 15min at 37°C. The DNA was again purified by phenol extraction and ethanol precipitation described above. To remove the single-stranded terminal regions and create blunt ends the DNA was digested with 1 unit of mung  
35 bean nuclease (Pharmacia, North Ryde, Australia) for 10 min at 37°C in buffer containing Na acetate (30mM), pH4.6,

NaCl (50mM) and ZnCl<sub>2</sub> (1mM) before extraction with phenol/chloroform and recovery by ethanol precipitation. Finally the DNA was digested with BamHI (Pharmacia) and the fragments were separated by electrophoresis in low-melting-point agarose. The BamHI A and D fragments were excised, recovered by NACS column chromatography (BRL, Gaithersburg, Md) and ligated with BamHI/HincII-cut plasmid Bluescribe M13<sup>+</sup> (Stratagene, La Jolla, Ca) prior to transformation into *E. coli* JM109. Positive clones carrying fragments of the expected size were identified, restriction digested and confirmed as correct by nucleotide sequencing and comparison with partial sequence determined directly from genomic DNA. This revealed that three 3'-terminal nucleotides were removed during the cloning procedure.

#### Nucleotide Sequencing of the OAV287 Genome

The complete sequence of the OAV287 genome was determined by sequencing the BamHI fragments A-F using the Sanger method (25) and various kits provided by commercial suppliers. Nested deletions were constructed for the five largest fragments using a double-stranded nested deletion kit (Pharmacia). These were sequenced using standard primers. Based on newly determined sequence other nucleotide primers were synthesised using a DNA synthesizer (ABI, Model 391). In this way both strands of the entire genome and the junctions between the fragments were sequenced.

#### Mutagenesis of the OAV287 genome

For the construction of a full length OAV287 clone and subsequent modification of it to create plasmids such as pOAV200 and pOAV600 certain mutations were required. A relevant portion of the genome was subcloned into Bluescribe (Stratagene, La Jolla, Ca) or a similar plasmid which allowed rescue of single stranded DNA. Later it became possible to use dsDNA for mutagenesis. Oligonucleotides of the desired sequence were synthesized,

phosphorylated and used as primers as described by the manufacturers of Muta-gene Phagemid (Biorad Labs, Ca) or Altered sites II (Promega, Wi) mutagenesis kits. Mutations were generally identified by digestion with the appropriate restriction enzyme or by nucleotide sequencing, or both. Genome fragments containing introduced mutations were subcloned to create larger plasmids such as pOAV200 using appropriate unique restriction sites.

10 Construction of a Full-Length Genomic Clone of OAV287

The terminal BamHI A and D fragments (cloned in Bluescribe M13<sup>+</sup>) were each modified by mutagenesis to add the nucleotides lost during cloning and a KpnI site. The last base of the KpnI site incorporated the C at the 5' end of each genomic ITR sequence. This produced plasmids pAK and pDK (Figure 6).

The left hand approximately 21.5kb of the genome was constructed from the BamHI D and B fragments and the SphI A fragment of approximately 13kb. The genomic BamHI B fragment cloned in pUC13 was modified by mutagenesis (GCATGC to GCATCC) to remove the SphI site at position 8287 producing pUC13B. The modified fragment was released by BamHI digestion and cloned into pDK which had been cut with BamHI and dephosphorylated. Colonies carrying the recombinant plasmid pDBM (Figure 6) were identified by screening with an oligonucleotide which spanned the BamHI B/D junction. The SphI A fragment (approximately 13kb) was cloned into the SphI site of pSELECT (Promega) to form pSESPH. This fragment contains a SmaI site near its left hand end which is common to pDBM. The KpnI/SmaI fragment from pDBM was subcloned into pSESPH which had also been cut with KpnI/SmaI to produce pSELLH, a plasmid based on pSELECT which now contained the left-hand approximately 21.5kb of OAV287 DNA.

35 The right-hand end of the genome was constructed from pAK which contains the right-hand approximately 8.6kb

of the genome and overlaps the SphI A fragment. pAK was cut with SalI and ligated with SalI-cut pACYC184, a plasmid of 4.24kb which contains a gene encoding chloramphenicol (Cm) resistance and an origin for DNA replication, to form a pACm (Figure 6). This plasmid was cut with SphI and KpnI to produce the right-hand genomic fragment incorporating the pACYC184 sequences. This was ligated with the left-hand KpnI/SphI fragment of approximately 21.5kb prepared from pSELLH to produce the final plasmid pOAV287Cm (Figure 6). This plasmid replicates stably in E. coli and therefore removes the need to propagate the virus to obtain genomic DNA for further study. The recombinant genome in plasmid pOAV287Cm differs from the wild-type viral genome by the single point mutation in the SphI site (base 8287), by the presence of pACYC184 sequences in the SalI site and by the addition of a GTAC sequence between the ITRs. However, insertion of pACYC184 sequences in the SalI site disrupts two significant open reading frames whose functions are unknown. If either of the gene products was essential for replication, then pOAV287Cm could not produce infectious virus following transfection. To circumvent this potential problem pOAV287Cm was modified further. First, plasmid Bluescribe M13- (Stratagene, La Jolla, Ca.) was cut with HindIII and end-filled. The linear plasmid was then cut with SmaI, blunt-end ligated and transformed. The resulting plasmid contained an ampicillin resistance gene and origin of replication and lacked SalI and SphI sites but retained a unique KpnI site. This plasmid was cut with KpnI and ligated with KpnI-cut pOAV287Cm. Plasmids which were doubly resistant to ampicillin and chloramphenicol were selected and grown. One of these was cut with SalI to release the pACYC184 sequences, religated and transformed. The resulting plasmid pOAV100 contained the AmpR gene and replication Ori inserted in the KpnI site between the ITR's of the genome (Figure 7). This

plasmid replicated stably in E. coli strain JM109 when maintained in the presence ampicillin (200µg/ml). Large quantities of plasmid were grown for transfection studies. Transfection of DNA and Virus rescue

5 To determine whether the recombinant genomic clone was infectious, pOAV100 was cut with KpnI to release the linear viral genome and DNA was transfected into CSL503 sheep foetal lung cells using lipofectamine (GibcoBRL). Solution (A) containing plasmid DNA (2-10µg) and 300µl  
10 EMEM (containing hepes + glutamine), but lacking foetal calf serum (FCS) and solution (B) containing lipofectamine (10µl) + 300µl EMEM (containing hepes + glutamine), but lacking FCS were combined, mixed gently and incubated for 45 minutes at room temperature. Subconfluent CSL503 cells  
15 in a 60mm petri dish were rinsed with 3ml EMEM (plus hepes and glutamine) lacking FCS. EMEM (plus hepes and glutamine) but lacking FCS (2.4ml) was added to the mixture of solutions A and B, mixed gently and added to the rinsed CSL503 cells. Cells were incubated for 5 hours  
20 at 37°C in 5% CO<sub>2</sub>. The incubation medium was changed using complete EMEM plus FCS (10%) and cells were incubated at 37°C in 5% CO<sub>2</sub> until virus plaques or cytopathic effect was visible (7-15 days).

To confirm that viruses rescued from transfection of  
25 pOAV100 and pOAV200 were derived from those plasmids a portion of the genome of wild-type OAV287, OAV100 and OAV200 viruses was amplified by PCR. For OAV100 a primer pair spanning the region of the mutated SphI site at bases 8287-8292 was used. For OAV200 the primer pair spanned  
30 the insertion site for the ApaI/NotI polylinker between the pVIII and fiber genes. Wild-type OAV287 DNA was amplified as a control in each case. DNA amplified from wild-type OAV287 was cut with SphI whereas the DNA amplified from OAV100 was not (Figure 8A). Similarly  
35 OAV200 DNA was cut with ApaI, EcoRV and NotI whereas

OAV287 DNA was not (Figure 8B). Other viruses were similarly characterised by restriction enzyme digestion. Identification of MLP/TLS elements and Construction of pMT

OAV287 TLS elements were identified as follows and  
5 as described (17). mRNAs present in OAV287-infected  
CSL503 cells were copied into cDNA by reverse  
transcription using primers complementary to the IIIa or  
fiber genes. A primer thought to fall within TLS exon 1  
was then paired with each cDNA primer for PCR. DNA was  
10 successfully amplified, cloned and sequenced. This  
identified TLS exons 2 and 3 (which correspond to bases  
8083-8145 and 8350-8412 of Figure 1, respectively) and the  
3' boundary of TLS exon 1 which occurs at base 5044 of  
Figure 1. A second PCR strategy was then used to obtain  
15 MLP and TLS fragments suitable for assembly into pMT. The  
region in Figure 1 between nucleotides 4861 and 5023,  
thought to contain the MLP was amplified by PCR using a  
plus sense primer which added an ApaI sequence at the 5'  
end and a 3' minus sense primer which introduced an NdeI  
20 site by point mutation at base 5012. Similarly, the TLS  
was amplified using a plus sense primer which introduced  
the NdeI site at base 5012 and a minus sense primer which  
was complementary to bases 8396-8412 and which added a  
HindIII site at the 3' end of the PCR product. The PCR  
25 fragments were digested with ApaI/NdeI and NdeI/HindIII,  
respectively and the fragments were cloned into Bluescript  
SK+ (Stratagene) cut with ApaI/HindIII. The resulting  
plasmid was then digested with HindIII/NotI and a  
synthetic oligonucleotide with HindIII/NotI termini and  
30 the sequence shown in Figure 9 was cloned to produce  
plasmid pMT. Genes of interest were then cloned into  
convenient restriction sites in the NCS. Gene expression  
cassettes were subcloned as ApaI/NotI fragments into  
pOAV200 or rescued into infectious virus.

### Infection of cells and expression of antigens

CSL503 and other cells were infected with viruses at a multiplicity of infection of 20pfu/cell as described previously (21). Infection was allowed to proceed for 24-  
5 60 hr. Cells were then incubated in methionine-free medium in the presence of  $^{35}\text{S}$ -methionine to label newly synthesized proteins. The protein of interest was recovered from cell lysates by immunoprecipitation using a specific antiserum against the expressed protein (21).  
10 Recovered proteins were analysed by polyacrylamide gel electrophoresis and detected by autoradiography or using a phosphorimager (Molecular Dynamics).

### RESULTS

To characterise the genome in molecular terms, BamHI  
15 restriction fragments representing the entire OAV287 genome were cloned into various plasmids and sequenced using methods described in Sambrook (9) and similar publications. Sequences were determined on both strands by using nested sets of deletion mutants together with  
20 synthetic oligonucleotide primers which were synthesized from newly determined sequences.

The viral sequence of 29,544 nucleotides (Figure 1) is considerably shorter (by approximately 6.5kb) than the sequence for human adenoviruses but many genes encoding  
25 structural proteins are identified by their homology with their Ad2 homologues (Figure 2). It is clear, however, that the ovine adenovirus genome shows major structural and sequence variations compared with all other adenoviruses studied to date (Figure 2), in the regions  
30 encoding both structural and non-structural proteins. In particular,

(a) the reading frames tentatively identified as forming the E1A/B regions are named principally on the basis of their location in the genome. Very limited  
35 homology can be detected between the 44.5kD open reading frame (orf) and the large T E1B protein of other

adenoviruses. Homology in the putative E1A region of OAV287 has not so far been detected;

5 (b) in other adenoviruses the E4 region is normally located at the right-hand end of the genome. The OAV287 E4? region is tentatively identified based only on the presence of a protein sequence motif HCHC..PGSLQC which is found in 18.8kD and 30.85kD orfs in this region. Identical or very similar motifs are found in the E4 34kD protein of human Ad2 and Ad40 and mouse adenoviruses;

10 (c) the distance between the end of pVIII and the beginning of fiber, which in other viruses defines the E3 region, is only 197 nucleotides (Figure 4). The E3 region equivalent, if it exists in ovine adenovirus, may consist of the cluster of open reading frames which are present in  
15 the right to left orientation on the complementary DNA strand, at the right-hand end of the genome (Figures 2 and 5). However, these sequences show no detectable homology with any other adenovirus and the functions of these proteins cannot be deduced from such comparisons;

20 (d) there is a region of approximately 1kb which lies between E3? and E4? which has a very high A/T content (70.2%) (Figure 1). As there are no open reading frames encoding greater than approximately 30 amino acids in length on either DNA strand it is unlikely that the region  
25 codes for any proteins, unless mRNAs are generated by very complex splicing events. This region has no known equivalent in any other adenovirus;

(e) other differences are apparent in the structural proteins of the virus. OAV287 lacks homologues of Ad2  
30 proteins V and IX. However, OAV287 has a completely new gene coding for p28kD which is located on the complementary strand of the E1A? region (Figure 2 and 3). This is a structural protein with an apparent size of 28kD by SDS PAGE which, according to N-terminal sequencing  
35 data, is cleaved from a larger precursor. No homology



between this protein and others in the databases has been detected;

(f) in most other genomes the VA RNA genes are located between the Terminal protein and the 52/55k genes. In OAV287 there is no room for them as the reading frames overlap.

These differences serve to emphasize the unique character of the OAV287 isolate compared with other human and animal adenoviruses. In addition, since the OAV287 non-structural regions show little or no homology with equivalent regions in other adenoviruses, sequence comparisons do not reveal the identity of likely non-essential regions of the genome. Moreover the viral DNA cannot easily be manipulated to test for dispensable sequences.

The present inventors have produced a plasmid containing a full length infectious copy of an ovine adenovirus genome in which the ITR sequences are linked by a short sequence which creates a unique restriction enzyme site. A plasmid containing a full length infectious copy of an ovine adenovirus genome linked to a bacterial origin for DNA replication and a marker gene has been produced. Partial clones of OAV287 genomic DNA were specifically modified and initially linked to a gene encoding antibiotic resistance and origin of replication inserted into the unique SalI site of the genome (Figure 6 and see Methods). Such a plasmid can be grown in bacteria and more easily manipulated.

The circular genomic clone differs from the naturally occurring circles that occur in Ad5-infected cells (10) and that might exist in OAV287-infected cells in that the 40 base pair ITRs are joined by a GTAC linker. Together with the last and first nucleotides of the genome (G and C, respectively, see Figure 1), this sequence forms a unique KpnI site (GGTACC) when the ITRs are joined head to tail. Other sites such as EcoRI, BamHI, SalI, Kasi etc

which have recognition sequences beginning with G and ending with C are suitable if they are unique as the 3' and 5' terminal nucleotides of other adenovirus genomes are G and C, respectively. A plasmid with a suitable antibiotic resistance gene e.g. ampR and origin of replication can be inserted at the unique site or elsewhere in the genome to form a plasmid which can be propagated in bacteria. Plasmids propagated in the presence of 200 µg/ml ampicillin in *E.coli* strains JM109 and DH5-alpha retain the KpnI sites and inserted sequences, indicating that the OAV287 ITR sequences are stable when linked in this manner. This approach may therefore be used to engineer other adenovirus genomes. If desired the GTAC linker sequence can be removed and the authentic termini regenerated prior to transfection by digestion with KpnI (or another appropriate enzyme) and incubation with T4 DNA polymerase to create blunt ends (9).

A method for generating linear infectious genomes from circular plasmids involved digesting the circular plasmid containing the full length copy of the OAV287 genome with restriction enzyme KpnI to generate a genome with the authentic 5' nucleotide dCMP. The linear DNA is then introduced into CSL503 cells using lipofectamine as the transfecting reagent.

To develop a viral genome as a vector it is essential to identify region(s) of the genome which are non-essential for function. These regions can be then substituted or deleted to make room for foreign DNA (11, 12), or they may be the site for insertion of foreign DNA. In the human adenovirus genome DNA has been substituted or inserted into the E1 and E3 regions (13, 14, 15) and at the extreme right-hand end of the genome between E4 and ITR, usually with the concomitant deletion of non-essential regions to facilitate packaging of the genome (16). Adenoviruses will package genomes up to ~6% larger

than the wild-type, probably due to physical constraints dictated by the capsid structure (11).

Non-essential sites in the OAV287 genome were identified by insertion of a polylinker sequence  
5 containing ApaI and NotI restriction sites. This linker was introduced into the genome copy in pOAV100 between nucleotides 22,139 and 22,130 of Figure 1 by site directed mutagenesis to create plasmid pOAV200 (Figure 7). This corresponds to a site located in the intergenic region  
10 between genes for the pVIII and fiber proteins which was chosen because it avoids disruption of RNA processing signals in the region. A transcription termination site for the L4 family of RNAs maps 26 nucleotides upstream and the splice junction between  
15 the tripartite leader sequences and fiber mRNA maps 144 nucleotides downstream of the insertion site, respectively (17). Transfection of pOAV200 into CSL503 cells resulted in the rescue of virus OAV200. The second site at which the polylinker was inserted was located between bases  
20 26,645 and 26,646 of Figure 1. This created plasmid pOAV600 (Figure 7). This insertion site corresponds to the right hand end of the A/T-rich region (Figure 2) whose function and precise boundaries are unknown. The site was chosen as it is six nucleotides to the left of the  
25 transcription termination point for RNAs transcribed from right to left from the E3' region (Figure 2). This was determined by sequencing cloned RT-PCR-amplified cDNAs derived from the region using methods similar to those described for the pVIII/fiber region (17). Transfection  
30 of pOAV600 into CSL503 cells yielded virus OAV600.

The above insertion strategy identified two regions of the genome which can be interrupted and created sites for subcloning gene expression cassettes.

A further non-essential site was identified using  
35 the unique SalI site located at bases 28644-28649 of Figure 1. The site was cut with SalI, end-filled and

religated to disrupt the reading frames which spanned the site. A plasmid pOAV600S (Figure 7), which had lost the site was identified by digestion with SalI. When pOAV600S was transfected into CSL503 cells, virus OAV600S was recovered. The loss of the SalI site in this virus was confirmed by digesting the viral genome with SalI. As the SalI site falls within two significant open reading frames (which extend on the complementary strand between bases 28457 and 29014 and between 28511 and 28699), which were disrupted by end-filling and religation, the gene products derived from the reading frames are probably also dispensable. This group of reading frames may therefore constitute the E3 region of OAV287 as no other gene products in any adenovirus are dispensable for replication, in vitro. This implies that it should be possible to delete the whole region labelled as E3? in Figure 2. In addition, in other experiments a 1kb NdeI fragment was deleted from the region marked as E4? in Figure 2. This deletion disrupted several reading frames in the region. No virus has been rescued from a such a plasmid, suggesting that it is not dispensable and accordingly, it may be E4.

Many viruses replicate incompletely in heterologous hosts, often entering cells but being unable to produce mature virus particles because of a block in the replication cycle. In the context of recombinant viral vectors, this represents a desirable safety feature, provided that replication is not blocked before appropriate and effective expression of the foreign gene occurs. OAV287 does not replicate productively in heterologous cell types (18), the only exception so far being bovine nasal turbinate cells in which viral titres are significantly reduced compared with the CSL503 cells. Recombinant forms of OAV287 have been constructed to determine whether expression of a reporter gene under the control of an appropriate promoter occurs.

Foreign gene expression requires that the gene be functionally linked to a promoter. This may be a viral promoter inherent in the genome, or a foreign promoter subcloned together with the gene of interest into a suitable site. The promoter driving gene expression must function in CSL503 and preferably a range of other cell types. In this work an OAV287 genomic promoter was used initially. Subsequently an heterologous promoter was also used. In adenoviruses, expression of the structural proteins is driven by the major late promoter (MLP). Families of RNA transcripts derived from the MLP contain a common sequence element, the tripartite leader sequence (TLS) at their 5' ends. The present inventors have identified those nucleotides in the OAV287 genome which comprise the TLS by using RT-PCR amplification of late mRNA transcripts present in OAV287-infected cells and sequencing of cloned cDNAs (17). A candidate MLP was expected to be present just to the left of TLS exon 1 (Figure 2). The MLP and TLS elements were subcloned using PCR techniques into a separate plasmid pMT (Figure 9) and linked with genes of interest. These promoter/gene cassettes were subcloned as *Apa*I/*Not*I fragments into the polylinker *Apa*I/*Not*I sites of pOAV200. Using this strategy plasmids pOAV203, pOAV204, pOAV205 and pOAV210 were constructed. These incorporate genes encoding a 17kD soluble protein from *T. colubriformis*, a rotavirus VP7sc gene (19), the 45W antigen from *Taenia ovis* (20) and a membrane protein (PM95) from *Lucilia cuprina*, respectively. Plasmid pOAV202, contained the 17kD antigen but lacked the MLP/TLS elements. These plasmids were transfected into CSL503 cells and rescued as viruses OAV202, OAV203, OAV204, OAV205 and OAV210, respectively (Figure 10).

The human cytomegalovirus immediate early IE94 promoter plus enhancer, which functions in a range of human and animal cell types (21), was also linked to the

rotavirus VP7sc antigen gene. This cassette was assembled by replacing the MLP/TLS elements in pMT/VP7sc with the HCMV enhancer-promoter region. The cassette was inserted in pOAV200 to create pOAV206. pOAV206 was transfected  
5 into CSL503 cells and virus OAV206 was rescued (Figure 10).

CSL503 and other cells were infected with the viruses described above and at various times post-infection the cells were radiolabelled with <sup>35</sup>S-methionine. Proteins of interest were recovered from cell  
10 lysates by immunoprecipitation using an appropriate antiserum. Recovered proteins were analysed by polyacrylamide gel electrophoresis and detected by autoradiography.

When virus OAV202 was used, no expression of the *T. coulbriformis* 17kD antigen was observed by immunofluorescence. As this virus lacks the MLP/TLS elements and carries only the 17kD gene this result demonstrates that there is no viral promoter upstream or  
15 adjacent to the insertion point between the pVIII and fiber genes which is capable of driving gene expression. As the E3 region is also missing from this site there is no requirement for a nearby promoter. This situation contrasts with observations made using a human Ad5 E3  
20 recombinant (21). In this case a promoterless gene inserted 3' proximal to the pVIII gene was expressed, probably from the adjacent E3 promoter or the upstream MLP (15, 21). This result further emphasizes the unique nature of the OAV287 genome. Recombinant OAV287 viruses  
25 carrying the MLP/TLS elements were tested for expression in CSL503 cells. With OAV204, expression was easily detected in infected, but not in uninfected cells at 24hr post-infection (Figure 11A). Similarly, when viruses  
30 OAV205, and OAV210 were tested, gene products of 24kD and approximately 95kD, respectively were detected (Figure  
35 11B). Therefore it is clear that MLP/TLS elements contain

the necessary information to drive gene expression in the homologous cell line under replication-permissive conditions. However, when OAV204 was tested in a heterologous rabbit kidney cell line in which the virus does not replicate productively, no VP7sc expression was observed. Some replication occurs in bovine nasal turbinate cells, although to a lower titre than in CSL503 cells. In the latter cells, expression of VP7sc was detected following infection with OAV204 (Figure 11B).

Virus OAV206 containing the HCMV enhancer/promoter element linked to the VP7sc gene was used to examine the function of a heterologous promoter in the context of the OAV287 genome. CSL503 cells infected with this virus readily expressed VP7sc antigen at 24-48hr post infection (Figure 12A). With this virus VP7sc expression was also observed in the non-permissive rabbit kidney cell line and in bovine nasal turbinate cells (Figure 12B). These results suggest that the HCMV or a similar constitutive promoter may be preferred over the MLP to drive gene expression in OAV recombinants in non-permissive cells.

One recombinant virus was also administered to sheep. Five sheep were vaccinated intraconjunctivally and intranasally with  $0.7 \times 10^8$  pfu of OAV203. At three days post-inoculation virus was recovered from the nasal swab of one sheep and from the conjunctival swabs of two sheep and confirmed as the recombinant virus by PCR analysis. Animals showed no obvious ill effects from such vaccination.

The viral vectors of the present invention can be used for the delivery and expression of therapeutic genes in grazing animals. In species which are not normally infected by ovine adenoviruses the lack of pre-existing immunity should allow efficient infection, gene delivery and expression. The genes may encode vaccine antigens, molecules which promote growth in production animals, molecules which modify production traits by manipulating

hormone responses and other biologically active or therapeutic molecules. The virus does not replicate productively in many non-ovine cells but the use of heterologous promoters allows the delivery and expression of genes while minimising the possibility of virus spread to a non-target host. As the DNA of adenovirus vectors can persist in cells in an unintegrated form, with the appropriate choice of promoter, expression over a prolonged period can be achieved.

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- 20 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all aspects as
- 25 illustrative and non-restrictive.